Crude Flavonoids from *Carya cathayensis* Sargent inhibited HeLa Cells Proliferation through Induction of Apoptosis and Cell Cycle Arrest

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SUMMARY. The aim of this study was to investigate the anticancer properties of crude flavonoids from *Carya cathayensis* Sargent bark (CCS-F), including the inhibitory effect of CCS-F on HeLa cells and the apoptosis-inducing capacity *in vitro*. In our results, treatment of HeLa cells with CCS-F resulted in the growth inhibition effect, and the IC_{50} was 95 μg/ml. Detection of apoptosis was performed by acridine orange / ethidium bromide (AO/EB) and Tdt-mediated dUTP nick end labeling (TUNEL) staining assays, which showed more apoptosis cells in CCS-F treatment group than the control group. Furthermore, CCS-F (100 μg/ml) could arrest the cells in G0/G1 phase. Meanwhile, the expression of Bax was increased in the cells treated with CCS-F (100 μg/ml), with an increase in the activity of caspase-3, while Bcl-2 expression was decreased compared to the control cells. It demonstrated that CCS-F had antiproliferative activity in HeLa cells and might be a potential anticancer drug.

INTRODUCTION

Our world is a rich source of medicinal plants and many plant extracts have been used against diseases in folk medicine, but only a few of these have been scientifically investigated. Plant-derived natural products such as flavonoids, alkaloids, polysaccharides, and so on have received considerable attention in recent years because of their various pharmacological properties, including cytotoxic and cancer chemopreventive effects. According to one report, over 50% of the drugs in clinical trials for anti-tumor activity were isolated from natural sources or are related to them [1].

Flavonoids, also called bioflavonoids, were a group of roughly 3,000 naturally occurring phenolic compounds sharing a similar chemical structure. They were found in every family and nearly every species of higher plants, and thus were in almost all fruits, vegetables, and medici-

nal herbs. Flavonoids could be classified into six categories: Flavanones, Flavones, Isoflavones, Flavonols, Flavanols, Anthocyanidins and proanthocyanidins [2]. Some flavonoids have been the subject of a large number of *in vitro* studies and a small number of *in vivo* ones, including genistein [3-5], luteolin [6], apigenin [7,8], quercetin [9], daidzein [10,11], and so on. Flavonoids were capable of inhibiting cancer cells through multiple means and did have potential in cancer treatment based on the many *in vitro* studies and the few *in vivo* studies available. In the future, some flavonoids would be better characterized than alkaloids or polysaccharides in the field of natural anticancer drugs and more researches would identify those having the greatest potential anticancer.

Carya cathayensis Sargent was a widely occurring plant species in some regions and it had several popular names according to the place

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where it was found. The nut of it was rich in many nutrient ingredients, and its nut exocarp had radioprotective, immunological and antitumor effects on mice [12,13]. In China the stem-bark of *Carya cathayensis* Sargent was used as folk medicine by indigenous people to treat a variety of illnesses including cancer, tinea, pain, inflammation, rheumatism and erysipelas [14]. Some reports have shown that the bioactive compounds of arbor always presented in the stem bark [15-20]. The same genus plant *Carya illinoensis* was also reported that hexane bark extracts of it processed antimycobacterial activity [21]. Phytochemical analyses of *Carya cathayensis* Sargent bark revealed a broad chemical constitution, and it was previously believed that this fact could explain its use. The main compounds already isolated from the plant bark were gallic acid, betasitosterol, betulin, anthraquinones and triacontanoic acid [22]. The effects of flavonoids from bark of *Carya cathayensis* Sargent on cancer cells have not been researched, although its nut exocarp has been studied in part [12,13].

Taking these findings into account, the present work was carried out to unravel the anticancer activity of flavonoids from *Carya cathayensis* Sargent against the HeLa cells.

MATERIALS AND METHODS
Chemicals

Fetal bovine serum (FBS), RPMI-1640, penicillin G, and streptomycin were from GIBICO BRL., [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide] (MTT), dimethylsulfoxide (DMSO), acridine orange, ethidium bromide and RNase A were purchased from Sigma Chemical Co. (St Louis, MO, USA). In Situ Cell Death Detection Kit and Caspase-3 Fluorometric Immunosorbent Enzyme Assay Kit were obtained from Roche Diagnostics (Mannheim, Germany). Rabbit monoclonal antibody to Bax and Bcl-2 were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Other chemicals used were of analytical grade.

Sample preparation and extraction

Carya cathayensis Sargent was purchased from Qinhuangdao Limin Pharmaceuticals Inc. (Hebei province, China) and authenticated by Prof. Zhao Jian-cheng, the botanist at Hebei Normal University, China. Voucher specimen (E061) was deposited at the herbarium under the location stated above. Powdered *Carya cathaensis* Sargent was extracted with 95% ethanol in the proportion of 1:8 (w/v) for 2 h

and repeated twice, and then the ethanol extracts was concentrated with a rotary evaporator (RE-52AA, Shanghai, China) and lyophillized (Thermo Savant, USA). The extracts was dissolved in water and extracted with petroleum ether. Subsequently, the defatted fraction was extracted with ethyl acetate and then gave the crude flavonoids. Finally, the crude flavonoids were purified with polyamide chromatography, and 95% ethanol was used as eluent during the process of polyamide chromatography. Then the eluent was collected and lyophilized to give refined flavonoids. The yield of refined flavonoids was 1.12% (w/w) and named as CCS-F. CCS-F was prepared as a stock of 1 mg/ml in basal medium RPMI-1640 and kept at -20 °C.

The qualitative analysis of CCS-F was carried out with $NaBH_4$, 1%$FeCl_3$-ethanol and 1% NaOH reagent (these three reagents were giving positive tests for detecting the presence of flavonoids) [23] and the color of solution showed red, green and yellow repectivetly, which revealed that CCS-F was mainly consisted of flavone, flavonol and flavanones. And CCS-F might contain several other polyphenol compounds.

Cell cultures

HeLa cell line, the human cervical cancer cells, was obtained from the Cell Center of Chinese Academy of Medical Sciences. Human peripheral blood lymphocytes (HPBMC) from healthy volunteers by venipuncture, were isolated by lymphocyte separation medium (Histopaque 1077; Sigma Chemical Co., St. Louis, MO) density gradient centrifugation. Both HeLa cells and normal cells lymphocytes were incubated in RPMI-1640 medium containing 10 % FBS, 100 U/ml penicillin, and 100 µg/ml streptomycin at 37 °C and 5% CO_2 atmosphere.

MTT assay

The cytotoxicity assay was performed according to the MTT colorimetric assay, which was based on the conversion of MTT to formazan crystals by mitochondrial dehydrogenases. Brief, the cells were seeded within a 96-well culture plate ($1x10^4$ cells/well). After 24 h incubation, the cells were treated with different concentrations of CCS-F (30, 50, 70, 90, 110, 150 µg/ml) for 48 h. Absorbance in control and drug-treated wells was measured by an Automated Microplate Reader (Multiskan MK3, Finland) at 570 nm. The cytotoxicity of CCS-F and normal human lymphocytes was expressed as

IC$_{50}$ (concentration of 50% cytotoxicity, which was extrapolated from linear regression analysis of experimental data).

AO/EB staining assay

HeLa cells were treated with CCS-F (100 µg/ml) for 48 h, and then labeled using the nucleic acid-binding dye mix of 100 µg/ml acridine orange and 100 µg/ml ethidium bromide in phosphate buffered solution (PBS). The cells were examined by fluorescence light microscope (Nikon, Japan). Cells were distinguished according to the fluorescence emission and the morphological aspect of chromatin condensation in the stained nuclei. (1) Viable cells have uniform bright green nuclei with organized structure. (2) Early apoptotic cells have green nuclei, but perinuclear chromatin condensation is visible as bright green patches or fragments. (3) Late apoptotic cells have orange to red nuclei with condensed or fragmented chromatin. (4) Necrotic cells have a uniformly orange to red nuclei with organized structure [24].

TUNEL staining assay

Apoptosis was also determined by assessment of TUNEL staining assay in parallel to AO/EB staining using the In situ Cell Death Detection kit, according to the manufacturer's instructions. HeLa cells were treated with or without CCS-F (100 µg/ml) for 48 h, and then fixed in 10% neutral buffered formaldehyde for 10 min. After rinsing slides with PBS, the slides were incubated with blocking solution (3% H$_2$O$_2$ in methanol) for 10 min at room temperature. The slides were rinsed with PBS, and then incubated slides in permeabilisation solution (0.1% Triton X-100 in 0.1% sodium citrate) for 2 min on ice. TUNEL reaction mixture was added on the samples after rinsing the slides with PBS, subsequently incubated for 60 min at 37 °C in a humidified atmosphere in the dark. Finally, a fluorescence microscopy (Nikon, Japan) was used to identify the apoptotic cells nuclei, as indicated by their distinct green color change. The number of cells undergoing apoptosis was counted in ten equal-sized fields and expressed as a percentage of the total number of HeLa cells in the same field.

Caspase-3 activity assay

Caspase-3 activity was analyzed using a fluorometric immunosorbent enzyme assay kit according to the manufacturer's instructions. Briefly, caspase-3 from cellular lysates (2x10^6 cells) was captured by a monoclonal anti-caspase-3 antibody precoated to the microtiter plate. Following the washing step, added the fluorogenic 7-amino-4-trifluoromethyl coumarin (AFC)-conjugated substrate substrate (AC-DEVD-AFC) was cleaved proportionally to the amount of activated caspase-3. Generated free fluorescent AFC was determined fluorometrically at 505 nm. Results are reported as formed AFC (µM/L) as determined against a standard curve.

Cell cycle analysis

HeLa cells (2x10^6) were seeded in 6-well culture plate and treated with CCS-F (100 µg/ml) for 48 h. Both floating and attached cells were collected, and poured together in the centrifuge tubes. Cells were washed with PBS, re-suspended and fixed in 70 % ice-cold ethanol for 1 h at 4 °C. Subsequently they were treated with RNase A for 30 min. Finally, cells were analyzed in a FACScan flow cytometer (Becton Dickinson, USA). The distribution of cells in the different phases of the cell cycle was analyzed from the DNA-histograms using CELLQuest software.

Analysis of Bcl-2 and Bax protein

The level of Bcl-2 and Bax protein was measured by flow cytometry as described with minor modifications [25]. Briefly, the cell samples were collected and washed with ice-cold PBS. After permeabilization with 0.5% Triton X-100, the cells were incubated with primary antibodies against Bcl-2 or Bax for 1 h, respectively, followed by incubation with corresponding fluorescein isothiocyanate (FITC)-conjugated secondary antibodies for 30 min at room temperature in the dark. After the cells were washed with PBS, the antigen density was analyzed by flow cytometry.

Statistical analysis

The data were expressed as mean ± standard deviation (SD). A one-way analysis of variance (ANOVA) using Origin7.0 software and $p < 0.05$ was considered statistically significant.

RESULTS
Cytotoxicity of CCS-F to HeLa cells

For most anticancer agents, cytotoxicity is measured by a standard MTT assay following a brief drug exposure. HeLa cells and normal human lymphocytes were treated with various concentration of CCS-F for 48 h. The results showed that the IC$_{50}$ of CCS-F towards HeLa cells was 95 µg/ml. On the other hand, The IC$_{50}$

of CCS-F towards normal human lymphocytes was 350 µg/ml. The cytotoxic activities of CCS-F were substantially lower towards normal human lymphocytes. On the basis of the results, CCS-F (100 µg/ml) was used in the next experiments with 48 h incubation.

Analysis of apoptosis induced by CCS-F

To determine the contribution of cell death to CCS-F-induced reductions in cell growth, we employed two different methods to detect apoptotic cells: AO/EB staining and TUNEL labeling. As shown in Figure 1, the majority of untreated cells was viable and displayed normal cellular morphology and intact nuclear architecture, with nuclei that showed uniform bright green. In comparison with controls, HeLa cells treated with CCS-F displayed both early and late features of apoptosis, as determined by enhanced fluorescence of condensed chromatin. These findings were confirmed by TUNEL assay, in which labeled uridine bases are attached to the DNA nicks characteristic of apoptotic cells showing green fluorescence. Many cells were apoptotic compared with the control group. Moreover, most of the cells counted apoptotic showed stronger intensity of green in the HeLa cells by treatment of CCS-F (100 µg/ml) (Fig. 2).

Distribution of cell cycle

We next explored the mechanisms underlying HeLa cells growth inhibition by CCS-F. The cell cycle distribution of cells treated with or without CCS-F was determined by fluorescence-activated cell sorting analysis of propidium iodide-stained cells, a measure of DNA content. As shown in Table 1, a higher proportion of cells was displayed in G0/G1 phase (47.1% *vs* 32.9%), and a concomitant reduce in S phase (25.4% vs 41.8%) by treatment of CCS-F (100 µg/ml) compared with the control group (p < 0.01).

Analysis of caspase-3 activity

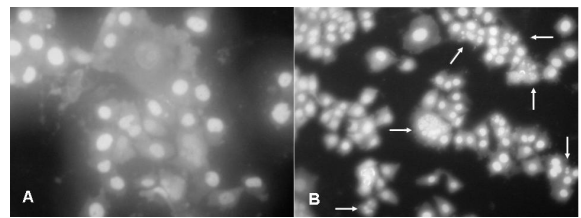To investigate potential molecular mechanisms for CCS-F-induced HeLa cells apoptosis,

Figure 1. AO/EB staining assay. **A**: the control group (x100). **B**: CCS-F (100 µg/ml) treatment group (x100); arrows show fluorescence of condensed chromatin and apoptotic body.
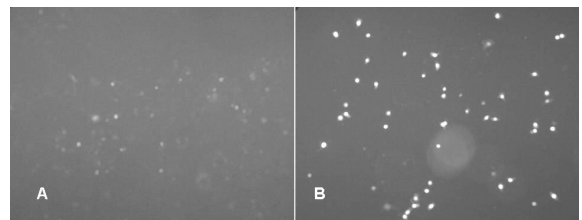

Figure 2. TUNEL staining assay. **A**: the control group (x100). **B**: CCS-F (100 µg/ml) treatment group (x100).
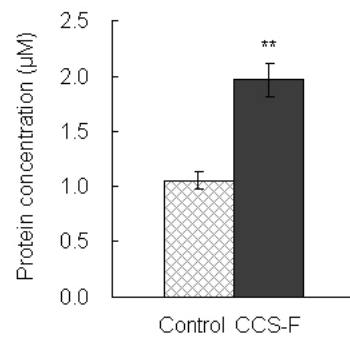

Figure 3. Caspase-3 activity assay. Caspase-3 activity was compared in HeLa cells treated with CCS-F (100 µg/ml) for 48 h vs the control group using an immunosorbent assay. Compared with the control group, statistical significance was ** p < 0.01.

we examined the effect of CCS-F (100 µg/ml) on cell caspase-3 activity, which was the one of apoptosis regulatory proteins. As shown in Figure 3, caspase-3 activity of cells by treatment of CCS-F was markedly elevated comparing with the control group (p < 0.01).

Group	G0/G1	S	G2/M
Control	32.9 ± 1.37	41.8 ± 1.24	25.3 ± 1.77
CCS-F (100 µg/ml)	47.1 ± 0.92**	25.4 ± 1.56**	27.5 ± 1.68

Table 1. Effect of CCS-F on distribution of cell cycle. Results are expressed as mean ± SD. Significant differences compared with the control, ** p < 0.01.

Group	Bcl-2 (%)	Bax (%)	Bcl-2/Bax value
Control	59.2 ± 3.15	7.5 ± 1.33	9.11
CCS-F (100 µg/ml)	45.8 ± 3.96*	19.1 ± 3.65*	2.40

Table 2. Effect of CCS-F on the expression of Bcl-2 and Bax proteins of HeLa cells. Results are expressed as mean ± SD. Significant differences compared with the control, * $p < 0.05$.

Expression analysis of apoptosis-regulated genes

We further revealed the mechanisms of CCS-F-induced HeLa cells apoptosis by analysis of Bcl-2 and Bax proteins expression. Comparing with the control group, the expression of Bax gene was increased and the expression of Bcl-2 was reduced by treatment of CCS-F (100 µg/ml) (Table 2, $p < 0.05$).

DISCUSSION

Flavonoids seem to be active constituents in numerous medicinal plants, and plants containing them are widely used in herbal medicine traditions around the world. As a whole, flavonoids tend to improve capillary resistance, inhibit inflammation, scavenge free radicals, and inhibit a variety of enzymes. The inhibition of cancer cell growth and induction in cell death were two major means to defeat tumor growth. In our results, data from MTT assay and cell apoptotic assessment demonstrated that CCS-F, the bioactive compound isolated from *Carya cathayensis* Sargent, inhibited proliferation and induced apoptosis of HeLa cells. Moreover, the IC50 values showed that the HeLa cell line was much more sensitive to CCS-F than normal human lymphocytes, so a relative low toxicity might be expected for CCS-F in further research.

We have indicated that the flavonoids of *Carya cathayensis* Sargent could inhibit the growth of HeLa cells and induce HeLa cells to undergo apoptosis. Apoptosis was a complex process involving a cascade of reactions and multiple genes, and it resulted in cytoskeletal disruption, cell shrinkage, membrane blebbing, internucleosomal DNA fragmentation and nuclear condensation [26]. Caspases, the cytoplasmic aspartate-specific cysteine proteases, played an important role in apoptosis [27]. Caspase-3 was one of the key executioners of apoptosis and responsible for the proteolytic cleavage of many critical proteins during apoptosis, activating the apoptotic processes [28]. The proteolytic activities of caspase-3 controlled the occurrence and de-velopment of apoptosis, and were considered the central part of apoptosis [29]. In this study, CCS-F (100 µg/ml) could elevate significantly the expression of caspase-3. It indicated that stimulation of the caspase-3 expression might have contributed to apoptosis increase observed in the CCS-F treated HeLa cells.

In addition to caspase-3, the Bcl-2 family of proteins, as the pivotal regulators of the mitochondrial apoptotic pathway, was involved in positive and negative regulation of apoptotic cell death. Among the numbers of Bcl-2 family, a major regulation of the apoptotic death signal resided with the Bcl-2 and Bax genes. Bcl-2 was an apoptosis-suppressing and Bax was an apoptosis-promoting protein [30]. It was clear that Bcl-2 or Bax may control the mitochondrial permeability transition pore or other early mitochondrial perturbation. Bcl-2/Bax ratio in a cell acted as regulating its own susceptibility to apoptosis [31]. In our results, Bcl-2 expression was reduced in the HeLa cells, while Bax expression was increased, contributing to the increment of apoptosis in the CCS-F treatment cells.

Cancer cells were no different from normal cells in their need to be stimulated before entering the cell cycle, except that stimulation was excessive in cancer cells. This excessive stimulation provided a number of targets for inhibiting cancer cell proliferation. In addition to preventing proliferation, keeping cells from entering the cell cycle might have the long-term effect of inducing apoptosis. When cells were not able to divide, they eventually died of apoptosis in most cases [2]. Thus, control of cell cycle progression in cancer cells was considered to be a potentially effective strategy for the control of tumor growth as the molecular analyses of human cancers [32]. In the study, treatment of HeLa cells with CCS-F could affect processes in the cell cycle proper, arresting in the G0/G1 phase. Hence, one mechanism of anticancer effect of CCS-F could be an inhibition of the malignant proliferation of cancer cells.

CONCLUSION

Our data suggested that CCS-F might act as an inhibitor of Hela cells proliferatin *in vitro* and be a potential, natural apoptosis-inducing anticancer agent. Meanwhile, the crude flavonoids of *Carya cathayensis* Sargent needed to be further isolated and characterized the structure of an active compound, including animal antitumor studies.

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